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FORMULATION CONTAINING STABLE GRANULAR LEUKOCYTE COLONY STIMULATING FACTOR

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Specification

1. Title of the invention

FORMULATION CONTAINING STABLE GRANULAR LEUKOCYTE COLONY STIMULATING FACTOR

2. Claims

1. A formulation containing a stable granular leukocyte colony stimulating factor, characterized by including a granular leukocyte colony stimulating factor as an active ingredient and including at least one kind being selected from a group comprised of pharmaceutical allowable amino acid, sulfur-included reducing agent, and antioxidant.

2. The formulation containing a stable granular leukocyte colony stimulating factor of Claim 1, characterized by the fact that at least one kind selected from the above-mentioned group comprised of amino acid, sulfur-included reducing agent, and antioxidant is included at an amount of 1-10,000 parts by weight

[1] Numbers in the margin indicate pagination in the foreign text.

to the granular leukocyte colony stimulating factor at 1 part by weight.

3. The formulation containing a stable granular leukocyte colony stimulating factor of Claim 1, characterized by the fact that the above-mentioned amino acid is at least one kind selected from a group comprised of glycine, threonine, tryptophan, lysine, hydroxylysine, histidine, arginine, cysteine, and methionine.

4. The formulation containing a stable granular leukocyte colony stimulating factor of Claim 1 or 2, characterized by the fact that the above-mentioned sulfur-included reducing agent is at least one kind selected from a group comprised of N-acetylcysteine, N-acetylhomocysteine, thioctic acid, thiodiglycidyl, thioethanolamine, thioglycerol, thiosorbitan, thioglycollic acid and its salt, sodium thiosulfate, sodium hydrogen sulfite, sodium pyrosulfite, sodium sulfite, thiolactic acid, dithiothreitol, glutathione, and mild sulfur-included reducing agent having a sulfuhydryl group having 1-7 carbon atoms.

5. The formulation containing a stable granular /2 leukocyte colony stimulating factor of Claim 1 or 2, characterized by the fact that the above-mentioned antioxidant is at least one kind selected from a group comprised of

erysorbic acid, dibutylhydroxytoluene, butylhydroxyanisole, dl-α-tocopherol, L-ascorbic acid and its salt, L-ascorbic plamitate, L-ascorbic stearate, triamyl gallate, propyl gallate, disodium ethylenediaminetetraacetate (EDTA), and polyphosphate.

3. Detailed explanation of the invention

(Industrial application field)

The present invention pertains to a formulation containing a granular leukocyte colony stimulating factor. In particular, the present invention pertains to a formulation containing a granular leukocyte colony stimulating factor in which the loss, inactivation, or decomposition of active ingredients during the storage are favorably prevented and the active ingredients are stabilized.

(Prior art)

Recently, in chemical therapies of various kinds of infectious diseases, resistant strain generation, alternative phenomenon of etiogenic bacteria, or high side effect are clinically serious problems, and for this reason, there is a movement for solving the fundamental problems of the above-mentioned chemical therapies by using substances for activating the defense functions of an infectious disease host apart from the above-mentioned chemical therapies such as antibiotics and

antibacterial agents. In other words, for example, it is considered that the deficient feeding and sterilizing action of leukocytes among the defense functions of the host has a mostly strongly influence at the initial stage of the bacterial infection. Accordingly, it is considered that it is important to realize the sthenia of the infection defense function of the host by stimulating the multiplication, differentiation, and maturation of neutrophils. As one of very useful substances showing such an action, there is a granular leukocyte colony stimulating factor (G-CSF), and the infection defense agent using it has already been filed for a separate patent by this applicant (Japanese Patent Application No. Sho 60[1985]-23777).

(Problems to be solved by the invention)

As mentioned above, in various kinds of chemical therapies, there are various kind of unavoidable problems, and for this reason, an attempt of using substances capable of activating the defense function of a body being infected, that is, a host is made.

Needless to say, it has been clarified that the G-CSF has an activity that activates the defense function of the host in itself and is also very useful in combination with the above-mentioned chemicals to further sufficiently exert the clinical therapeutic effect.

The G-CSF is used at a very infinitesimal amount, and a formulation containing 0.1-500 μg (preferably 5-50 μg) G-CSF per one adult is usually dosed at a ratio of 1-7 times/week. However, the G-CSF is unstable and easily influenced by external factors such as temperature, humidity, oxygen, and ultraviolet rays. As a result, physical and chemical changes such as association, polymerization, or oxidation and decomposition are caused, so that the activity is largely decreased. This means that not only the therapeutic action for very accurately dosing a very infinitesimal amount of G-CSF cannot be completely carried out, but an estimated loss portion of the active ingredients must be added extra into the chemicals.

Accordingly, it is necessary to solve these problems and to develop a product that can sufficiently prevent the activity of active ingredients from being decreased. In other words, the purpose of the present invention is to provide a formulation containing a stable G-CSF.

(Means to solve the problems)

According to the present invention, the stability of the above-mentioned intended formulation containing a G-CSF was variously reviewed and researched to improve it. As a result, it was discovered that the addition of pharmaceutical allowable amino acid, sulfur-included reducing agent, antioxidant, or

these mixture was effective. Then, the present invention was completed.

In other words, the formulation containing a stable G-CSF of the present invention is characterized by including a granular leukocyte colony stimulating factor as an active ingredient and including at least one kind being selected from a group comprised of pharmaceutical allowable amino acid, sulfur-included reducing agent, and antioxidant.

Also, the G-CSF being used in the present invention, for example, can be obtained according to various methods described in the specifications of Japanese Patent Application Nos. Sho 59[1984]-153273, Sho 60[1985]-269455, Sho 60[1985]-269456, Sho/3 60[1985]-270838, and Sho 60[1985]-270839 that have already been filed. For example, a human G-CSF can be obtained by preparing a recombinant DNA using a gene for coding the human G-CSF through the culture of a cell strain (CNCM deposit Nos. "I-315" and "I-483") sampled from a tumor cell of a patient with an oral cavity bottom cancer and manifesting it by an appropriate host cell (for example, ovarian cells of colon bacillus, C-127 cell, and Chinese hamster).

As the G-CSF in the present invention, any human G-CSF purified at high purity can be used, and it is also obtained by isolating from a cultured supernatant fluid being obtained by

culturing a human G-CSF producing cell. Also, a polypeptide or glucoprotein having a human G-CSF activity being produced by a transformant being obtained by transforming a host by a recombinant vector in which a gene for coding a polypeptide having a human G-CSF activity is assembled is preferable.

Specifically, G-CSF shown by the following (i) and (ii) is especially preferably used.

(i) Human G-CSF having the following physiochemical properties.

(1) Molecular weight: About 19,000 ± 1,000 measured by a sodium dodecylsulfate-polyacrylamide gel electrophoresis method.

(2) Isoelectric point: Having at least one of three isoelectric points of pI = 5.5 ± 0.1, pI = 5.8 ± 0.1, and pI = 6.1 ± 0.1.

(3) Ultraviolet part absorption: Having a maximum absorption at 280 nm and a minimum value at 250 nm.

(4) An amino acid sequence from N terminal to the 21st residue is as follows.

H_2N-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-

(ii) Human G-CSF containing a polypeptide having a human granular leukocyte colony stimulating factor activity represented by the following amino acid sequence or its part or a glucoprotein having said polypeptide and a sugar chain part.

$(Met)_n$ Thr Pro Leu Gly Pro Ala Ser Ser Leu

Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln

Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu

Gln Glu Lys Leu $(Val\ Ser\ Glu)_n$ Cys Ala Thr

Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu

Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro

Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu

Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu

Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu

Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp

Thr Leu Gln Leu Asp Val Ala Asp The Ala Thr

Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met

Ala Pro Ala Leu Gln Pro Thr Gln Gly Ala Met

Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala

Gly Gly Val Leu Val Ala Ser His Leu Gln Ser

Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His

Leu Ala Gln Pro (However, m represents 0 or 1, and n represents 0 or 1.)

Also, for the detailed manufacturing method of these G-CSF, see the specifications of Japanese Patent Application Nos. Sho 59[1984]-153273, Sho 60[1985]-269455, Sho 60[1985]-269456, Sho 60[1985]-270838, and Sho 60[1985]-270839 that have already been filed by this applicant.

Also, as another method, it can be obtained by culturing a hybridoma being obtained by cell-fusing a G-CSF producing cell and a malignant tumor cell having a self-multiplication capability in the presence or absence of a mitogen.

The solution containing the human G-CSF obtained by these methods can be purified, enriched, and freeze-stored as needed by well-known means or can be stored after removing moisture by a means such as freeze-drying.

All the human G-CSF obtained in this manner can be changed to formulations containing a stable G-CSF by the present invention.

The amino acid as a stabilizer being used to obtain the formulation containing a stable G-CSF of the present invention is glycine, threonine, tryptophan, lysine, hydroxylysine, histidine, arginine, cysteine, and methionine. Also, as the sulfur-included reducing agent, N-acetylcysteine, N-acetylhomocysteine, thioctic acid, thiodiglycidyl, /4 thioethanolamine, thioglycerol, thiosorbitan, thioglycollic acid and its salt, sodium thiosulfate, sodium hydrogen sulfite, sodium pyrosulfite, sodium sulfite, thiolactic acid, dithiothreitol, glutathione, and mild sulfur-included reducing agent having a sulfuhydryl group having 1-7 carbon atoms can be mentioned. Furthermore, as the antioxidant, erysorbic acid,

dibutylhydroxytoluene, butylhydroxyanisole, dl-α-tocopherol, L-ascorbic acid and its salt, L-ascorbic plamitate, L-ascorbic stearate, triamyl gallate, propyl gallate, or chelating agent such as disodium ethylenediaminetetraacetate (EDTA), sodium polyphosphate, and sodium metaphosphate can be mentioned.

The formulation containing a stable G-CSF of the present invention includes at least one kind selected from these groups.

The amino acid, sulfur-included reducing agent, antioxidant or its mixture are preferably used at an amount of 1-10,000 parts by weight to the granular leukocyte colony stimulating factor at 1 part by weight.

Furthermore, the formulation containing a stable G-CSF of the present invention can include diluent, dissolution aid, isotonic agent, excipient, pH adjustor, pain killer, buffer agent, and adsorption preventive for its formulation.

The stabilized formulation containing G-CSF of the present invention can be used as an oral or parenteral dose type such as various kinds of injections and can be realized in various formulations in accordance with said dose types. For example, oral dose agents such as tablet, pill, capsule, granule, and suspension, or a solution such as vein injection, muscle injection, hyperdermic injection, and intradermic injection, suspension injection, freeze-dried agent, or mucous dose

formulation such as suppository, nasal agent, and vaginal agent can be mentioned as its typical formulations.

(Operation)

As mentioned above, in the chemical therapies of infectious diseases, in addition to chemical such as antibiotics and antibacterial agents, in order to simultaneously improve the defense functions themselves based on an immune response power such as resistance and activity of patients, active ingredients are added, which is a clinically very useful means.

The G-CSF as one of these components is used at a very infinitesimal amount. Therefore, in case the G-CSF is processed as an aqueous solution with a very low concentration, for example, it is frequently used by putting it into syringe, etc., or including it in a container such as ampoule. In such a case, the G-CSF is influenced by various kinds of external factors such as temperature, humidity, oxygen, and ultraviolet rays and subjected to association, polymerization, or oxidation. These physical and chemical changes largely lower the activity of the G-CSF.

Thus, it was recognized that it was difficult to maintain the effective concentration of the drug solution in the container or the target activity of the components in a prescribed unit dose. Therefore, in consideration of the amount

being lost due to the instability, it was necessary to pre-add the amount more than is necessary for the therapy.

Accordingly, in the present invention, the above-mentioned problem was solved by adding at least one kind selected from a group comprised of amino acid, sulfur-included reducing agent, and antioxidant in the formulation containing G-CSF. These additives can suppress the automatic oxidation speed being accelerated by the temperature and the humidity, for instance, or can prevent the association or polymerization based on them.

As the phenomenon being presumed from these results, since the G-CSF is a high-molecular protein, though its detailed reaction mechanism is not clear, the mechanism is effectively operated to stabilize the G-CSF. Such a problem is distinct in injection solution, suspension, etc., and it is also similarly seen in the formulation processes of other tablets, etc. The use of amino acid or sulfur-included reducing agent or antioxidant is also effective for this case.

With the addition of at least one kind being selected from these amino acid, sulfur-included reducing agent, and antioxidant, the G-CSF is largely stabilized, and as proven in the following application examples, the activity of the G-CSF can be effectively maintained over a long term. The reason for this is presumed that the G-CSF molecules are protected from /5

external factors by the use of the above-mentioned additives and the probability of association and polymerization between them is largely reduced.

For this reason, the lower limit of the amount of additive as at least one kind being selected from a group comprised of amino acid, sulfur-included reducing agent, and antioxidant or its mixture is critical, and as mentioned above, the amount is preferably in a range of 1-10,000 parts by weight to the G-CSF at 1 part by weight in the formulation containing the G-CSF.

As mentioned above, according to the present invention, since the stability of the G-CSF can be effectively maintained, very specific high effectiveness and activity of the G-CSF as a very infinitesimal amount of component can be used for therapy. Furthermore, since the waste of expensive component can be prevented, the product cost can be lowered.

(Application examples)

Next, the present invention is explained in further detail by application examples. However, the present invention is not limited to the following examples.

Also, the residual activity of the G-CSF was measured as follows in the following application example.

(a) Soft agar method using a mouse bone marrow cell

0.4 ml house serum, 0.1 ml sample, 0.1 ml C3H/HeN (female) mouse bone marrow cell floating liquid (0.5-1 x 10^5 nuclear cell), and 0.4 ml modified McCoy 5A culture solution containing 0.75% agar were mixed, put into a plastic dish for a tissue culture with a diameter of 35 mm, solidified, and cultured for 5 days under the condition of 37°C, 5% carbonic acid gas/95% air, and 100% humidity, and the number of colonies formed (a group composed of 50 cells or more was assumed as one colony) was counted, and the activity for forming one colony was assumed as one unit.

Also, the "modified McCoy 5A culture solution" used in the above-mentioned method (a) was prepared as follows.

Modified McCoy 5A culture solution (two-time concentration):

12 g McCoy 5A culture solution (made by GIBCO Co.), 2.55 g MEM amino acid vitamin medium (made by Hisui Seiyakusha), 2.18 g sodium bicarbonate, and 50,000 units penicillin G potassium were dissolved twice in 500 ml distilled water, filtered and sterilized by a millipore filter of 0.22 μm, and used.

Application Example 1

A formulation (20 mM phosphoric acid buffer solution, containing 100 mM sodium chloride, pH 7.4) containing 50 μg/ml G-CSF in which additives shown in Table I were added to 50 μg G-CSF was aseptically prepared, and a freeze-dried formulation was

manufactured. The change of the G-CSF activity with time was measured by the soft agar method using the above-mentioned mouse bone marrow cell (a). The results are shown in Table I. Also, the activity (%) in the table is a relative ratio to the initial unit and defined by the following expression.

Activity (%) = activity unit after a lapse of prescribed time/initial activity x 100

The freeze-drying conditions are as follows.

The G-CSF solution to which a stabilizer was added was put into an aseptic sulfur-treated glass vial, frozen at - 40°C or lower for 4 h, and primary-dried at a temperature of - 40-0°C and the degree of vacuum of 0.03-0.1 Torr for 48. Then, it was secondary-dried at a temperature of 0-20°C and the degree of vacuum of 0.03-0.08 Torr for 12 h, and the vial inside was substituted by an aseptic dry nitrogen gas until reaching an atmospheric pressure. Then, the vial was nailed with a rubber plug for freeze-drying and sealed with an aluminum cap.

Table I /6

添加剤	添加量（重量部）	活性（%）4℃6ヶ月保存後	活性（%）37℃1ヶ月保存後
トリプトファン	1000	89	88
ヒスチジン	1000	98	94
システイン	1000	95	85
チオグリコール酸ナトリウム	200	92	86
チオ硫酸ナトリウム	200	89	87
dl－α－トコフェロール	100	97	92
ジブチルヒドロキシトルエン	20	91	90
L－アスコルビン酸ナトリウム	100	97	92
無添加	—	74	58

1. Additive
2. Amount added (parts by weight)
3. Activity (%) after storing at 4°C for 6 months
4. Activity (%) after storing at 37°C for 1 month
5. Tryptophan
6. Histidine
7. Cysteine
8. Sodium thioglycollate
9. Sodium thiosulfate
10. dl-α-tocopherol
11. Dibutylhydroxytoluene

12. Sodium L-ascorbate

13. No addition

Application Example 2

A formulation (20 mM phosphoric acid buffer solution, containing 100 mM sodium chloride, pH 7.4) containing 10 μg/ml G-CSF in which additives shown in Table II were added to 50 μg G-CSF was aseptically prepared, aseptically filled into a sulfur-treated glass vial, and sealed, so that a formulation containing a G-CSF solution was manufactured. For these solution formulations, the change of the G-CSF activity with time was measured similarly to the method of Application Example 1, and the results were shown in Table II.

Table II

添加剤	添加量（重量部）	活性 %		
		4℃7日間保存後	4℃2ヵ月間保存後	室温1ヵ月保存後
トリプトファン	100	98	94	93
ヒスチジン	100	96	90	90
システイン	100	98	97	94
チオグリコール酸ナトリウム	100	97	92	87
チオ硫酸ナトリウム	200	99	90	87
dl－α－トコフェロール	100	98	90	84
L－アスコルビン酸ナトリウム	100	97	88	80
ジブチルヒドロキシトルエン	50	93	93	91
トリプトファン チオ硫酸ナトリウ	100 100	98	95	92
無添加	—	89	71	57

1. Additive
2. Amount added (parts by weight)
3. Activity (%)
4. After storing at 4°C for 7 days
5. After storing at 4°C for 2 months
6. After storing at room temperature for 1 month
7. Tryptophan
8. Histidine
9. Cysteine
10. Sodium thioglycollate
11. Sodium thiosulfate
12. dl-α-tocopherol

13. Sodium L-ascorbate

14. Dibutylhydroxytoluene

15. Tryptophan

    Sodium thiosulfate

16. No addition

(Effects of the invention)

As mentioned above in detail, according to the present invention, with the use of a prescribed amount of at least one kind being selected from amino acid, sulfur-included reducing agent, and antioxidant, the problems such as loss of active ingredients and decrease of the activity resulting from the association, polymerization, or oxidation based on external factors such as temperature, humidity, oxygen, and ultraviolet rays of G-CSF existing at a very infinitesimal amount in the formulation can be effectively solved.

Therefore, the amount of G-CSF being dosed to patients can be very accurately administered and controlled, and the expensive G-CSF can be effectively utilized. Thus, the cost of the formulation containing the G-CSF can also be reduced.